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Evaluation of Glutathione Peroxidase 3 Protein Expression in Clear Cell and Other Ovarian Carcinomas by Immunohistochemistry

Fatemeh Nili[1*], Dorsa Ghasemi[2], Ramtin Edjtemaie[3], Fereshteh Ameli[2]

1. *Department of Pathology, Faculty of Medicine, Imam Khomeini Hospital Complex, Tehran University of Medical Sciences, Tehran, Iran*
2. *Department of Pathology, Imam Khomeini Hospital Complex, Tehran University of Medical Sciences, Tehran, Iran*
3. *Department of Pathology, Faculty of Medicine, Guilan University of Medical Sciences, Rasht, Iran*

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Corresponding Information:
Fatemeh Nili,
Department of Pathology, Faculty of Medicine, Imam Khomeini Hospital Complex, Tehran University of Medical Sciences, Tehran, Iran

Email: f_nili@yahoo.com

ABSTRACT

Background & Objective: Clear cell carcinoma (CCC) is an uncommon subtype of ovarian cancer, with inherent resistance to common chemotherapy agents. Glutathione peroxidase 3 (GPX3) is one of the important mechanisms of cell defense against cell damage signals. Recent studies have suggested that suppression of the GPX3 gene increases sensitivity to Cisplatin chemotherapy drugs against ovarian clear cell carcinoma (OCCC). The present study was conducted with aim to evaluate glutathione peroxidase 3 protein expression in clear cells and other ovarian carcinomas by immunohistochemistry.

Materials & Methods: Patients with the diagnosis of ovarian carcinoma, who were referred to the pathology department of Imam Khomeini Hospital, were included. Immunohistochemical staining for GPX3 was performed on the paraffin blocks. The intensity and percentage of the marker were evaluated.

Results: A total of 76 patients, including 31 CCC, 33 high-grade serous carcinomas (HGSC), 10 endometrioid carcinomas, and 2 low-grade serous carcinomas were examined. The mean age of the patients was 52.06 ± 11.4 years. The mean age in the CCC group (49.9±9.6 years) and in the non-clear cell carcinoma (NCCC) group (53.5±12.5 years) was not significantly different (P=0.1). Most of the patients in the CCC group were stage 1 (63%), while in the NCCC group, most of the patients (61%) were in stage 3 (P=0.000). After immunohistochemical staining, no protein expression was seen in any of the CCC and NCCC tumoral samples.

Conclusion: Immunohistochemistry is not a sensitive method to evaluate GPX3 protein expression. Studies in this field should be done by molecular or serologic evaluations.

Keywords: Clear Cell Carcinoma, Ovary, Glutathione Peroxidase, Immunohistochemistry

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1. Introduction

Ovarian cancer is the most common cause of death from genital tract cancers in women (1-3). Ovarian clear cell carcinoma (OCCC) accounts for 5-25% of ovarian carcinomas (4-6). The mean age of patients is at 5 and 7 decades (7, 8). Among all ovarian epithelial cancers, this tumor is most related to pelvic endometriosis, para-endocrine hypercalcemia, and venous thromboembolism (5, 8, 9). Clear cell carcinoma can be seen in the endometrium, fallopian tubes, cervix and vagina.

Microscopically, this tumor shows various tubulocystic, solid and papillary patterns. Cytologically, the most common cell types are clear and hobnail cells. Clear cells are polygonal, with distinct cell borders. These cells contain cytoplasmic glycogen. Hobnail cells contain dark and bulbous nuclei (4, 9). To diagnose OCCC, the presence of clear cells alone is not enough. Immunohistochemistry (IHC) may be necessary for accurate diagnosis. Immunohistochemistry is a kind of special staining, which specifically identify tissue proteins. The principle of the staining method is binding commercial antibodies to specific proteins of biological tissues as antigens. After exposure to a chromogenic substrate, the antibodies are labeled with enzymes which produce specific color. In IHC study, OCCCs express epithelial markers including keratin, Cytokeratin corresponds to Moll's peptide (CAM5.2), Cytokeratin 7 (CK7) and Epithelial membrane antigen (EMA). Cancer Antigen 125 (CA125) and Vimentin are positive in 50% of

cases, while Alpha fetoprotein (AFP), CK20, P53, Cluster of differentiation 10 (CD10) and Carcino-embryonic Antigen (CEA) are negative **(4, 9)**. Napsin A is expressed as a specific marker in ovarian clear cell carcinoma **(10, 11)**. Wilm's Tumor (WT1) is expressed in 10% of cases and Thyroid Transcription Factor 1 (TTF1) in less than 20% of cases **(12)**. Estrogen Receptor (ER) and Progesterone Receptor (PR) are usually negative **(13)**. Hepatocyte Nuclear Factor 1β (HNF1β) and Alpha Methyl CoA Racemase (AMACR) are also sensitive and specific marker for the diagnosis **(14, 15)**. The differential diagnosis for ovarian clear cell carcinoma include dysgerminoma, yolk sac tumor, juvenile granulosa cell tumor, Kruckenberg's tumor and metastatic renal clear cell carcinoma **(4)**.

OCCC is recognized as a histological type with a poor prognosis in advanced stages. This tumor has a poor response to chemotherapy with Cisplatin **(7, 8)**.

Considering the significant difference between OCCC and other ovarian epithelial cancers in terms of prognosis, pattern of malignant recurrence and response to chemotherapy, it is needed to accurately differentiate this tumor from other ovarian malignancies and discover biomarkers for targeted therapy of these patients **(16-20)**.

Glutathione peroxidase (GPX3) is an important cytoplasmic antioxidant enzyme, and its main function is to neutralize reactive oxygen species (ROS). This enzyme is one of the important mechanisms of cell defense against cell damage signals. Recent studies showed overexpression of this gene in OCCC **(18, 21)**. Other studies suggested that suppressing the GPX3 gene increases the sensitivity to Cisplatin drugs in in OCCC **(22)**. Expression of this marker is also considered as a prognostic factor in response to treatment **(22)**. Since there are limited studies conducted on the expression level of this protein in OCCC, the present study was conducted with aim to evaluate glutathione peroxidase 3 protein expression by IHC in OCCC and compare it with other types of carcinomas.

2. Materials and Methods

This retrospective cross-sectional study was performed through examining the archives of Imam Khomeini Hospital's pathology department, the clinical data and slides of patients diagnosed with malignant ovarian carcinoma who did not receive neoadjuvant treatment before surgery and were admitted to this center between 2015-2019 and had a suitable paraffin block. Considering 54% sensitivity and 46% specificity of the biomarker, 95% confidence interval and 8% error, the sample size of 70 specimens was determined.

Due to the low prevalence of clear cell carcinoma and the possibility of reducing the number of these diagnosis, a group of clear cell cases and a non-clear cell carcinoma (NCCC) group were selected. After examining the microscopic slides by two gynecopathologists, the appropriate block was selected. In order to perform IHC evaluations after cutting, unstained sections with a thickness of three microns from the paraffin blocks, antigen retrieval and immunohistochemical staining were performed on all samples using the polymeric biotin-free horseradish peroxidase method for the GPX3 marker according to the instructions of the kit (Goat polyclonal antibody, 1/100, Abcam, ab10448, Cambridge, U.K). Secondary antibody (Master Diagnostica, Anti goat Master Polymer, Spain) addition and counterstaining with Hematoxilin were also done. Cytoplasmic protein expression was evaluated for intensity and percentage of stained tumor cells. Normal kidney tissue was used as positive control in all steps of IHC study.

Data were analyzed using Statistical Package for Social Science (SPSS) software (version 23). T-test or Mann-Whitney test was used to compare quantitative variables between the two groups. The qualitative variables were compared using Chi-square test or Fisher's exact test. $P<0.05$ was considered significant.

The study was approved by the Ethics Committee of Imam Khomeini Hospital (ethical code: IR.TUMS.IKHC.REC.1400.125).

3. Results

In this study, by exploring Imam Khomeini hospital database, because of rarity of the disease in the general population, only 31 cases of OCCC who successfully met inclusion criteria were found. A total of 76 patients including 31 clear cell carcinomas, 33 high-grade serous carcinomas (HGSC), 10 endometrioid carcinomas, and 2 low-grade serous carcinomas were investigated (Table 1).

In terms of clinical stage, most of the patients in the CCC group were stage 1 (63%), while in the NCCC group, which were more of the HGSC type, most of the patients (61%) were in stage 3, and the difference was statistically significant (P=0.000) (Table 2).

The mean age of the studied patients was 52.06 ± 11.4 years with a range of 27 to 78 years. The mean age in the CCC group was 49.9±9.6 years and in the NCCC group was 53.5±12.5 years; there was no statistically significant difference (P=0.1).

After immunohistochemical staining, the positive control sample (normal kidney tissue) showed positive staining in proximal tubules (Figure 1), but no protein expression was seen in any of the CCC and NCCC tumoral samples (Figure 2).

Due to the non-immunoreactivity in tumor cells, it was not possible to evaluate the sensitivity, specificity, and positive and negative predictive value, as well as the relationship with prognostic factors.

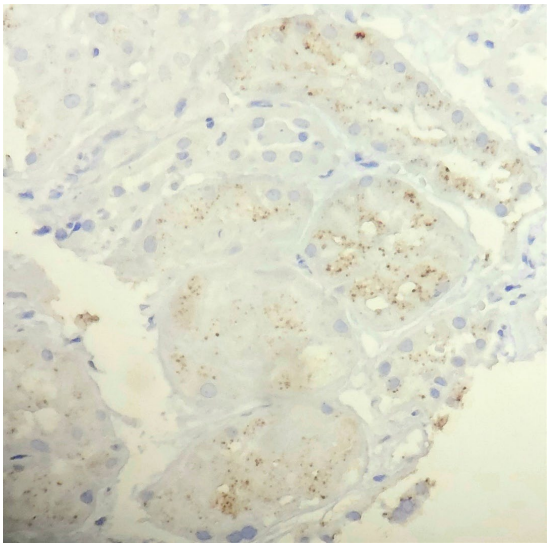


Figure 1. Cytoplasmic granular staining in proximal tubules of the kidney (positive control)(400X)

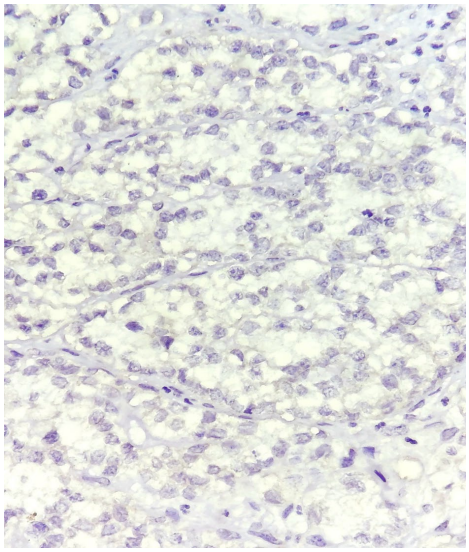


Figure 2. Negative staining in tumor cells (400X)

Table 1. Frequency of different histopathological subtypes of ovarian carcinoma

	Frequency	Percent
Endometrioid carcinoma	10	13.2
High grade serous carcinoma	33	43.4
Low grade serous carcinoma	2	2.6
Clear cell carcinoma	31	40.8
Total	76	100.0

Table 2. Pathologic stage of the patients with clear cell carcinoma (CCC) and Non-clear cell carcinoma (CCC)

Histologic subtype		stage				Total
		1	2	3	4	
CCC	17	2	5	3	27	
	63.0%	7.4%	18.5%	11.1%	100.0%	
Non CCC	8	7	25	1	41	
	19.5%	17.1%	61.0%	2.4%	100.0%	
Total		25	9	30	4	68
		36.8%	13.2%	44.1%	5.9%	100.0%

4. Discussion

Clear cell carcinoma is an uncommon subtype of ovarian carcinoma with lowest estrogene receptor (ER) and progesterone receptor positivity among malignant epithelial tumor of ovary **(13, 23)**. Most cases of the disease are detected in stage I which is limited to the ovary. In this stage, the prognosis is better than patients with high-grade serous carcinoma. However, in the case of the spread of extra-ovarian tumor spread and the need for chemotherapy, the prognosis is worse. The reason is the inherent resistance of these tumors to common chemotherapy drugs **(7)**.

In the present study, most of the OCCC were diagnosed at stage 1 of the disease. In a study in Japan (2006), Takano et al **(24)** studied 254 OCCC. The mean age of the patients was 52.4 years. Moreover, 13% of tumors had stage Ia, 36% stage Ic, 13% stage II, 30% stage III and 6% stage IV. The 5-year survival and overall survival were 84% and 88% in stage I, 57% and 70% in stage II, 25% and 33% in stage III, and 0 and 0 in stage IV **(24)**.

GPX is one of the antioxidant enzyme systems in mammalian cells. The role of reactive oxygen agents

and antioxidant enzymes in carcinogenesis and tumor progression complicates targeting of antioxidant enzymes as therapeutic approaches for cancer treatment **(25)**. Few studies have focused on the evaluation of GPX in ovarian carcinomas. In the present study, GPX3 protein expression was evaluated in clear cells and other subtypes of epithelial ovarian carcinomas. Unfortunately, in none of the cases, the protein expression was seen by IHC method. The first issue is accuracy of the IHC procedure and efficacy of the applied antibodies. Since the expression of GPX3 has been proven in the epithelial cells of the kidney proximal tubules, we used a kidney sample in all steps of IHC study to ensure the correctness of the procedure, the result of which was positive.

In a previous research conducted by Saga et al **(22)** in Japan, they investigated the role of GPX3 in ovarian clear cell carcinoma, based on DNA microarray analysis **(22)**. In a review study conducted in 2015 by Kobayashi et al **(18)** in Japan with the aim of identifying genes and immunohistochemical and serological markers in the diagnosis of clear cell carcinoma of the ovary and its differentiation from high grade serous carcinoma of the ovary, it was found that some genes and their products expressed in OCCC can be used as serum markers in the diagnosis of this disease **(18)**. They found that GPX3, Napsin Aspartic Peptidase A (NAPSA), and Aldehyde Hydrogenase 1 A1 (ALDH1A1) markers, which are overexpressed in OCCC, can be considered as new serum diagnostic markers **(18)**.

In most of the previous studies, molecular tests or evaluation of serum marker level were done. The only study which performed IHC along with messenger Ribonucleic Acid (mRNA) analysis on 40 cases of ovarian CCC and NCCC, only weak staining was obtained in CCC and none of the serous carcinomas were positive **(21)**. The results of studies using IHC method suggest that the further evaluations are required to be done on fresh specimens and more sensitive methods, including molecular analysis.

The sensitivity and diagnostic efficacy of antibody kits and clones in identifying small amounts of biomarkers is also important that can be evaluated in future studies.

Prognostic impact of GPX3 expression is a matter of interest in the field of oncology research and investigated in different organ cancers as well as ovary **(21, 25-34)**.

5. Conclusion

Immunohistochemistry is not a sensitive method to evaluate GPX3 protein expression. The results of studies using IHC method suggests that the further evaluations are required to be done on fresh specimens and more sensitive methods, including molecular analysis and serologic tests.

6. Declarations

Acknowledgments

We would like to thank the staff of the immunohistochemistry department (IKHC Cancer Institute) for their technical support.

Ethical Considerations

All procedures were performed in accordance with the 1964 Helsinki Declaration and its latest amendments. Patients' personal information, including identity, was never published anywhere (IR.TUMS.IKHC.REC.1400.125).

Authors' Contributions

F. Nili designed the study, reviewed the slides and wrote the manuscript. R. Edjtemaie and D. Ghasemi retrieved the cases by preparing the parrafin blocks. R. Edjtemaie, D. Ghasemi and F. Ameli reviewed the slides, and revised the manuscript. All authors approved the final manuscript.

Conflict of Interest

The authors declare no conflict of interest.

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